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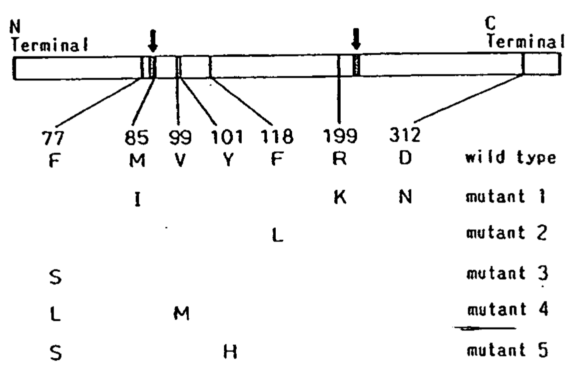
(54) Long-chain prenyl diphosphate synthase

(57) The present invention discloses a mutated enzyme comprising a geranylgeranil diphosphate synthase having its origin in wild type Sulfolobus acidocaldarius wherein, one of at least phenylalanine at position 77, methionine at position 85, valine at position 99, tyrosine at position 101, phenylalanine at position 118, arginine at position 199 and aspartic acid at position 312 is substituted with another amino acid.

Fig. 1



EP 0 763 542 A2

Description

BACKGROUND OF INVENTION

1. Field of Invention

The present invention relates to a mutant prenyl diphosphate synthase that is able to synthesize prenyl diphosphate having a longer chain than prenyl diphosphate synthesized by the native prenyl diphosphate synthase.

2. Related Art

Prenyl diphosphate is highly valuable in biosynthesis pathways, functioning as a precursor of steroids, a precursor of caratenoids, being a transition substrate of prenylated proteins, being a substrate for synthesis of vitamin E, vitamin K and ubiquinone (CoQ) and so forth. Prenyl diphosphate exists in various forms, including dimethylallyl diphosphate (DMAPP; C5), geranyl diphosphate (GPP; C10), farnesyl diphosphate (FPP; C15), geranylgeranyl diphosphate (GGPP; C20), geranylfarnesyl diphosphate (GFPP; C25), hexaprenyl diphosphate (HPP; C30), heptaprenyl diphosphate (HepPP; C35) and octaprenyl diphosphate (OPP; C40).

Prenyl transferases, which synthesize these prenyl diphosphates, are enzymes that form prenyl diphosphate by continuously condensing isopentenyl diphosphate (IPP; C5) into allylic diphosphate, and exist in various forms, including farnesyl diphosphate synthase (FPS), geranylgeranyl diphosphate synthase (GGPS), geranylfarnesyl diphosphate synthase (GFPS), hexaprenyl diphosphate synthase (HexPS), heptaprenyl diphosphate synthase (HepPS) and octaprenyl diphosphate synthase (OPS).

However, among the above-mentioned prenyl diphosphates, only those from dimethylallyl diphosphate having 5 carbon atoms to geranyl diphosphate having 20 carbon atoms are commercially available in small amounts as reagents, and a process for industrially synthesizing and recovering large amounts of prenyl diphosphates having longer chains is not known.

The carbon chain length and stereoisomerism of synthesized prenyl diphosphates are known to be specifically determined depending on the particular enzyme. Until now, it has not been clear what type of mechanism is the factor in determining carbon chain length.

Although prenyl transferases and their genes are known to be derived from bacteria, mold, plants and animals, these enzyme are typically unstable, difficult to handle and are not expected to be industrially valuable.

The prenyl transferases and their genes of thermophilic organisms, which are stable and easy to use as enzymes, are only farnesyl diphosphate synthase (FPS) (Koyama, T. et al. (1995) J. Biol. Chem. 113, 355-363) and heptaprenyl diphosphate synthase (HepPS) (Koike-Takeshita, A. et al. (1995) J. Biol. Chem. 270, 18396-18400) from the moderately thermophilic archaebacterium, Bacillus stearothermophilus; geranylgeranyl diphosphate synthase (GGPS) from the hyper thermophilic bacterium, Sulfolobus acidocaldarius (Ohnuma, S.-i. et al. (1994) J. Biol. Chem. 268, 14792-14797); as well as farnesyl diphosphate/geranylgeranyl diphosphate synthase (FPS/GGPS) from the methane-producing archaebacterium, Methanobacterium thermoautotrophicum (Chen, A. and Poulter, C.D. (1993) J. Biol. Chem. 268, 11002-11007). Only HepPS can synthesize prenyl diphosphate having 35 carbon atoms, and enzymes having thermal stability that synthesize prenyl diphosphates having 25 or more carbon atoms have not been reported. In addition, the abovementioned HepPS does not have adequate heat resistance, is composed of two types of subunits, and handling is not always easy.

SUMMARY OF INVENTION

Thus, the present invention provides a thermostable prenyl diphosphate synthase capable of synthesizing long-chain prenyl diphosphate, a process for its production, and a method for using said enzyme.

In order to create an enzyme that can synthesize prenyl diphosphate having a longer chain length, the inventors of the present invention succeeded in creating a mutant enzyme able to synthesize prenyl diphosphate having a longer chain than naturally-occurring geranylgeranyl diphosphate synthase by treating DNA coding for geranylgeranyl diphosphate synthase with a mutation agent, introducing the above-mentioned treated DNA into the yeast, Saccharomyces cerevisiae, deficient for hexaprenyl diphosphate synthase activity, and selecting a mutant DNA that can complement the above-mentioned deficient, and moreover, elucidated the relationship between the mutation site in the enzyme and the chain length of the prenyl diphosphate that is formed, thereby leading to completion of the present invention.

Thus, the present invention provides a mutant enzyme wherein, least one of phenylalanine residue at position 77, methionine residue at position 85, valine residue at position 99, tyrosine residue at position 101, phenylalanine residue at position 118, Arginine residue at position 199 and aspartic acid residue at position 312 in a geranylgeranyl diphosphate synthase of Sulfolobus acidocaldarius origin is substituted with another amino acid, and which enzyme can synthesize prenyl diphosphate having at least 25 carbon atoms.

Moreover, the present invention provides a gene system that codes for the above-mentioned enzyme, and a process for producing the above-mentioned enzyme using that gene system.

Furthermore, the present invention provides a process for producing a mutant prenyl diphosphate synthase comprising the steps of culturing a host transformed with a gene in which the codon for phenylalanine residue located at the fifth N-terminal side position from the N-terminal amino acid of the aspartate-rich domain I in a gene that codes for the native enzyme, is converted to a codon for a non-aromatic amino acid, thereby enabling the expression of a mutant enzyme that is able to synthesize prenyl diphosphates having a longer chain than the longest chain of prenyl diphosphate synthesized by the native prenyl diphosphate synthase.

In addition, the present invention provides a process for producing long-chain prenyl diphosphate using the above-mentioned enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 indicates the mutation site of the present invention in the geranyl diphosphate synthase derived from Sulfolobus acidocaldarius. The arrows in the drawing indicate two aspartate-rich domains.

Fig. 2 is photograph that indicates the autoradiograph of a thin layer chromatography which shows the products in the case of allowing the mutant enzymes of the present invention produced in yeast to act on substrates IPP and (all-E)-FPP. The ellipses show the positions of cold authentic samples, which are geraniol, farnesyl, and geranilgeranil for a, b and c respectively

Fig. 3 is a photograph that indicates the autoradiograph of a thin layer chromatography which shows the products in the case of allowing the mutant enzyme of the present invention produced in yeast to act on substrates IPP and (all-E)-GGPP. The ellipses show the positions of cold authentic samples, which are geraniol, farnesyl, and geranilgeranil for a, b and c respectively

Fig. 4 is a photograph that indicates the autoradiograph of a thin layer chromatography which shows the products in the case of allowing the mutant enzyme of the present invention produced in E. coli to act on (A) substrates IPP and DMAPP, and on (B) substrates IPP and GPP. The ellipses show the positions of cold authentic samples, which are geraniol, farnesyl, and geranilgeranil for a, b and c respectively.

Fig. 5 is the autoradiograph of a photograph that indicates a thin layer chromatography which shows the products in the case of allowing the mutant enzyme of the present invention produced in E. coli to act on (A) substrates IPP and (all-E)-FPP, and on (B) substrates IPP and (all-E)-GGPP. The ellipses show the positions of cold authentic samples, which are geraniol, farnesyl, and geranilgeranil for a, b and c respectively.

DETAILED DESCRIPTION

As a specific example in the present invention, a geranylgeranyl diphosphate synthase (GGPS) gene of the hyper thermophilic archaebacterium,

Sulfolobus acidocaldarius, is used for the starting material. The cloning method of this gene is described in detail in the specification of Japanese Patent Application No. 6-315572. In addition, another example for cloning the gene is described in the present specification as Example 1, and a nucleotide sequence and an amino acid sequence encoded thereby are shown as SEQ ID NO: 1.

In the present invention, a cloned DNA is mutated in vitro. Although chemical treatment using a mutagen, or physical treatment using UV light or X-rays can be used for the mutation means, chemical treatment is convenient to carry out. Any routinely used chemical mutagen can be used for the mutagenesis for the present invention, an example of which is nitrite.

A specific example of mutagenesis is shown in Example 2.

The mutagenized DNA is inserted into a yeast expression vector to prepare a DNA library. Any vector that is able to express an inserted extraneous gene in the yeast can be used as an expression vector, examples of which include a yeast plasmid such as pYEUra3 (available from Clonetech) and pYES2 (available from Invitrogen).

The resulting plasmid library is introduced into a yeast mutant strain defective for the ability to synthesize hexaprenyl diphosphate, which is one of the precursors of coenzyme Q6. Since this mutant strain is unable to synthesize coenzyme Q6 necessary for non-fermentative sugar metabolism, it cannot be grown in medium that contains glycerol as the sole carbon source. Thus, if the yeast transformed by the above-mentioned library is cultured in glycerol medium and the strains that grow are selected, strains can be selected that have acquired the ability to synthesize prenyl diphosphate having a large number of carbon atoms for coenzyme Q synthesis.

Five positive clones were obtained in this manner from approximately 1400 transformants. As a result of purifying the plasmids from these clones, determining the nucleotide sequence of the inserted fragment, and predicting amino acid sequences that are coded, each mutant had changes in the amino acid sequence as indicated below.

Mutant 1: Methionine at position 85 changed to isoleucine, arginine at position 199 changed to lysine, aspartic acid

at position 312 changed to Asn

Mutant 2: Phenylalanine at position 118 changed to leucine

Mutant 3: Phenylalanine at position 77 changed to serine

Mutant 4: Phenylanine at position 77 changed to leucine and valine at position 99 changed to methionine

Mutant 5: Phenylalanine at position 77 changed to serine and tyrosine at position 101 changed to histidine

In contrast to wild-type enzymes being unable to synthesize prenyl diphosphate having at least 25 carbon atoms, enzymes having amino acid sequences containing these changes were able to synthesize prenyl diphosphate having 25 or more carbon atoms Those amino acid sequences having the above-mentioned amino acid substitutions are shown in SEQ ID NOs: 2 to 6.

Thus, it can be logically surmised that if an amino acid at any one of the above-mentioned positions is replaced with another amino acid, a prenyl diphosphate having more carbon atoms than that synthesized by the native enzyme can be synthesized. Thus, the present invention provides a mutant enzyme in which at least one amino acid from among phenylalanine at position 77, methionine at position 85, valine at position 99, tyrosine at position 101, phenylalanine at position 118, arginine at position 199 and aspartic acid at position 312 is replaced with another amino acid, and said enzyme is able to synthesize prenyl diphosphate having at least 25 carbon atoms.

Particularly in the case that phenylalanine at position 77 is replaced with another amino acid, and preferably a non-aromatic amino acid such as serine or leucine, that enzyme is able to synthesize prenyl diphosphate having at least 25 carbon atoms. Thus, in one embodiment, the present invention provides an enzyme in which at least phenylalanine at position 77 is replaced with another amino acid such as serine, leucine or another non-aromatic amino acid This type at enzyme includes enzymes in which replaced amino acids are present at one or a plurality of the other above-mentioned positions. Examples at other amino acid positions include valine at position 99 and/or tyrosine at position 101.

Thus, the present invention includes enzymes in which only phenylalanine at position 77 is replaced, enzymes in which phenylalanine at position 77 and valine at position 99 are replaced, enzymes in which phenylalanine at position 77 and tyrosine at position 101 are replaced, enzymes in which phenylalanine at position 77, valine at position 99 and tyrosine at position 101 are replaced, and enzymes in which phenylalanine at position 77 and one or a plurality of amino acids at the above-mentioned positions are replaced.

According to another mode of the present invention, an enzyme in which methionine at position 85, arginine at position 199 and aspartic acid at position 312 are replaced with other amino acids is also able to synthesize prenyl diphosphate having at least 25 carbon atoms. Thus, the present invention, in another embodiment, includes an enzyme in which at least methionine at position 85, arginine at position 199 and aspartic acid at position 312 are replaced with other amino acids. In this embodiment, enzymes in which methionine at position 85, arginine at position 199 and aspartic acid at position 312 are replaced, as well as enzymes containing amino acid replacements at one or a plurality of sites other than at these sites or the above-mentioned mutation sites, are included.

According to still another embodiment of the present invention, an enzyme in which phenylalanine at position 118 is replaced with another amino acid can also synthesize prenyl diphosphate having at least 25 carbon atoms. Thus, in another embodiment, the present invention includes enzymes in which at least the amino acid at position 118 is replaced with another amino acid. In this embodiment, enzymes in which the amino acid at position 118 is replaced with another amino acid, as well as enzymes containing amino acid replacements at one or a plurality of positions of the above-mentioned amino acid replacement positions, are included.

Enzymes are known to have those own specificities of enzyme activities even in the case of being modified by addition, removal and/or replacement of one or a few amino acids. Thus, in addition to the peptides having the amino acid sequences shown in SEQ ID NOs: 2 to 6, the present invention also includes enzymes that the same specificity while having an amino acid sequence that is changed by replacing, deleting and/or adding one or a few, such as up to 5 or up to 10, amino acids with respect to the amino acid sequences shown in SEQ ID Nos: 2 to 6.

Two aspartate-rich domains (sites indicated with arrows in Fig. 1) are conserved in various prenyl transferases, and the diphosphate site of the substrate is thought to bind to these sites. Phenylalanine at position 77 exists at the 5th position upstream to the N-terminal side from the N-terminal of aspartate-rich domain I present on the N-terminal side among these two aspartate-rich domains. This phenylalanine is replaced with a non-aromatic amino acid in 3 of the 5 mutants of the present invention.

Thus, in order to synthesize prenyl diphosphate having a large number of carbon atoms, for example that having 25 or more carbon atoms, if phenylalanine at about the fifth position upstream to the N-terminal side from the amino acid of the N-terminal of aspartate-rich domain I is replaced with another amino acid, for example a non-aromatic amino acid, even in the case of a prenyl transferase other than the prenyl transferase derived from Sulfolobus acidocaldarius having the amino acid sequence indicated in Sequence No. 1, an enzyme is obtained that is able to synthesize prenyl diphosphate having a larger number of carbon atoms than the wild type enzyme.

Thus, the present invention provides a process for producing a mutant prenyl transferase characterized by replacing phenylalanine at the 5th position upstream to the N-terminal side from the amino acid of the N-terminal of aspartate-rich domain I of prenyl transferase. This amino acid replacement can be performed by changing the codon that codes

for that amino acid.

In addition, the present invention provides a gene coding for the various above-mentioned mutant enzymes, a vector comprising that gene, particularly an expression vector, and a host transformed with said vector. The gene (DNA) of the present invention can be easily obtained by introducing a mutation into DNA that codes for the wild type amino acid sequence indicated in SEQ ID NO: 1, according to routine methods such as site-directed mutagenesis or PCR.

Moreover, once the amino acid sequence of the target enzyme has been determined, a suitable nucleotide sequence that codes for it can be determined, thus making the mutant is possible to chemically synthesize DNA by conventional DNA synthesis methods.

In addition, the present invention provides an expression vector comprising the DNA as described above, hosts transformed with said expression vector, and a process for producing an enzyme or peptide of the present invention using these hosts.

Although expression vectors contain an origin of replication, expression control sequence and so forth, these vary according to the host, Examples of hosts include procaryotes, examples of which include bacteria such as E. coli and Bacillus sp. including Bacillus subtilus; eucaryotes, examples of which include yeasts such as Saccharomyces sp. including S. cerevisiae, and Pichia sp. including Pichia pastoris; molds, examples of which include Aspergillus sp. such as A. oryzae and A. niger; animal cells, examples of which include cultured cells and cultured cells of higher animals, such as CHO cells. In addition, it is also possible to use plants for the host.

According to the present invention, as indicated in Examples, geranylfarnesyl diphosphate can be accumulated in the culture by culturing a host transformed by the DNA of the present invention, and geranylfarnesyl diphosphate can be produced by recovering it from the culture. Also according to the present invention, geranylfarnesyl diphosphate can be produced by allowing the mutant GGPP synthase produced according to the process of the present invention to act on the isopentenyl diphosphate substrate and each allylic substrate such as farnesyl diphosphate.

In an example of using E. coli for the host, gene regulation of gene expression is known to exist such as in the process of transcribing mRNA from DNA and the process of translating protein from mRNA. In addition to those sequences present in nature (e.g. lac, trp, bla, lpp, P_L, P_R, ter, T3 and T7 as promoters), sequences in which their mutants (e.g. lacUV5) are artificially joined with wild type promoter sequences (e.g. tac, trc) are known as examples of promoter sequences that regulate mRNA transcription, and these can also be used in the present invention.

It is known that the ribosome binding site (GAGG and other similar sequences) sequence and the distance to the initiation codon are important as sequences that regulate the activity to translate the mRNA to synthesize proteins. In addition, it is also well known that the terminator, which commands termination of transcription on the 3'-end (e.g. a vector containing $rrnPT_1T_2$ is commercially available from Pharmacia), has an effect on protein synthesis efficiency in the recombinant.

Although commercially available products can be used as is for the vector that can be used for preparation of the recombinant vector of the present invention, various types of vectors induced according to a specific purpose can also be used. Examples of these include pBR322, pBR327, pKK223-3, pKK233-2 and pTrc99, originating in pMB1 and having the replicon, pUC18, pUC19, pUC118, pUC119, pBluescript, pHSG298 and pHSG396, modified to improve the number of copies, pACYC177 and pACYC184, derived from p15A and having the replicon, as well as plasmids originating in pSC101, Co1E1, R1 and F factor. Moreover, expression vectors, for fused proteins, that are easier to purify, can also be used, examples of which include pGEX-2T, pGEX-3X and pMal-c2, and the example of a gene used as the starting material in the present invention is described in Japanese Patent Application No. 6-315572.

In addition, gene introduction can also be performed by using virus vectors and transposons such as λ-phages and M13 phages in addition to plasmids. In the case of gene introduction into a microorganism other than E. coli, gene introduction into Bacillus sp. is known using puB110 (sold by Sigma) or pHY300PLK (sold by Takara Shuzo). These vectors are described in Molecular Cloning (J. Sambrook, E.F. Fritsch, T Maniatis ed., Cold Spring Harbor Laboratory Press, pub.), Cloning Vector (P.H. Pouwels, B.E. Enger Valk, W.J. Brammar ed., Elsevier pub.) and various company catalogs.

Insertion of a DNA fragment coding for GGPP synthase and, as necessary, a DNA fragment having the function of regulating expression of the gene of the above-mentioned enzyme, into these vectors can be performed according to known methods using suitable restriction enzyme and ligase. Specific examples of plasmids of the invention prepared in this manner include pBS-GGPSmut1, PBS-GGPSmut2, pBS-GGPSmut3, pBS-GGPSmut4 and pBS-GGPSmut5.

Examples of microorganisms that can be used for gene introduction with this type of recombinant vector include E. coli and Bacillus sp. This transformation can also be performed according to routine methods such as the $CaCl_2$ method or protoplast method described in Molecular Cloning (J. Sambrook, E.F. Fritsch, T. Maniatis ed., Cold Spring Harbor Laboratory Press pub.) and DNA Cloning Vol. I-III (D.M. Glover ed., IRL Press pub.).

In producing the mutant enzyme of the present invention, the above-mentioned transformed cell is cultured after which the mutant enzyme can be collected and purified from that culture in accordance with routine methods, examples of which include salting out, organic solvent sedimentation, gel filtration affinity chromatography, hydrophobic inter action chromatography and ion exchange chromatography.

In addition, the present invention provides a process for producing prenyl diphosphate using the enzyme of the present invention. In this process, the enzyme of the present invention should be allowed to react in a medium, and par-

ticularly an aqueous medium, and then the target prenyl diphosphate should be recovered from the reaction medium as desired. The enzyme may not only be purified enzyme, but also crude enzymes obtained by semi-purification through various stages, or a substance containing enzymes such as cultured microorganisms or the culture itself. In addition, the above-mentioned enzyme, crude enzyme or enzyme-containing substance may be an immobilized enzyme that has been immobilized in accordance with conventional methods.

Prenyl diphosphate having fewer carbon atoms than the target prenyl diphosphate, such as 5-20 carbon atoms and preferably less than 5 carbon atoms, and isopentyl diphosphate are used for the substrate. Water or an aqueous buffer, such as phosphate buffer, are used for the reaction medium.

EXAMPLES

The following Examples provide a more detailed explanation of the present invention. Furthermore, the materials used in the following Examples can all be easily acquired by a person with ordinary skill in the art as described below.

Strain C296-LH3 of the budding yeast, Saccharomyces cerevisiae (Tzagoloff, A. and Dieckmann, C.L. (1990) Microbiological Reviews 54, 211-255, Tzagoloff, A. et al. (1075) J. Bacteriol. 122, 826-831), was used for the screening host.

Plasmid pG3/TI (Tzagoloff, A. and Dieckmann, C.L. (1990) Microbiological Reviews 54, 211-255, Tzagoloff A. et al. (1975) J. Bacteriol. 122, 826-831, Ashby, M.N. and Edwards, P.A. (1990) J. Biol. Chem. 265, 13157-13164) or plasmid YEpG3ΔSpH, from which portions other than the HexPS coding region had been removed from pG3/TI (Ashby, M.N. and Edwards, P.A. (1990) J. Biol. Chem. 265, 13157-13164), was used for the positive control plasmid containing the HexPS gene.

Y-PGK, wherein the crtE gene portion had been removed from Y-crtE (Misawa, N. et al. (1990) J. Bacteriology 172, 6704-6712), was used for the expression vector for library preparation. Saccharomyces cerevisiae strain A451 was used as a wild strain used for the positive control.

However, the experimental materials required for the present invention are not limited to those described above, but rather completely similar substitutes can also be used.

Screening host mutant strain C2960-LH3 for screening is a deficient strain for the HexPS gene. In other words, a budding yeast HPS gene fragment can easily be obtained from a widely known wild strain of budding yeast by PCR using an already known budding yeast HexPS gene sequence (GenBank™/EMBL Data Bank accession number(s) JO5547). If this gene fragment is then used by coupling with a yeast incorporating plasmid (YIp) such as pRS403, pRS404, pRS405 or pRS406 (available from Stratagene), an HexPS-deficient strain can easily be created by widely conducted gene destruction using homologous recombination.

In addition, it also sufficient for the positive control plasmid if this gene fragment is inserted using a widely known budding yeast expression vector such as pYEUra3 (available from Clonetech) and pYES2 (available from Invitrogen). The strain used for the positive control is not limited to strain A451, but rather any strain is sufficient provided it retains the wild HexPS gene. In addition, it is sufficient to use a commercially available vector for the expression vector for library preparation such as pYEYra3 available from Clonetech or pYES2 available from Invitrogen.

LKC-18 reversed phase thin layer chromatography plates were purchased from Whatman Chemical Separation, Inc. [1-^{14}C]IPP was purchased from Amersham.

Example 1. Plasmid Construction

New HindIII restriction enzyme sites were introduced both upstream and downstream of the GGPS gene (GenBank™/EMBL Data Bank accession number D28748) of Sulfolobus acidocaldarius by PCR using the chemically synthesized DNA primers 5'-AAGAGAAGCTTATGAGTTACTTTGAC-3' (SEQ ID NO: 7) and 5'-GATACAAGCTTTATTTTCTCC-3' (SEQ ID NO: 8). Genomic DNA was purified in accordance with routine methods from Sulfolobus acidocaldarius, obtainable as ATCC33909 from the American Type Culture Collection (ATCC), and its clone DNA was then used for the template DNA of PCR.

The DNA fragment amplified with PCR was ligated to the HindIII site of plasmid pBluescript (KS$^+$) cleaved with HindIII to form pBS-GGPS. A crtE gene portion was removed by cleaving plasmid Y-crtE with HindIII, and the remaining portion containing the PGK promoter and PGK terminator was self-ligated to form Y-PGK. The insert portion containing GGPS gene obtained by severing pBS-GGPS with HindIII was introduced at the HindIII site of Y-PGK to form Y-GGPS.

Example 2. Random Mutagenesis of GGPS Gene

A random mutation was introduced into the region coding for GGPS gene using nitrite according to the method of Myers et al. (Myers, R.M. et al. (1985) Science 229, 242-247). Single strand DNA was isolated from E. coli containing pBS-GGPS by infection with helper phage M13K07, and this was then treated for 60 minutes with 1 M sodium nitrite. Next, the complementary strand was synthesized as primer using chemical synthesis DNA 5'-CCCCCCTCGAGGTC-

GACGGTATCGATAA-3' (SEQ ID NO: 9) corresponding to the sequence of the T7 promoter portion. The GGPS gene portion was then extracted with HindIII restriction enzyme, introduced at the HindIII site of Y-PGK, and transformed to E. coli strain XU-Blue to prepare the library.

Example 3. Yeast Transformation and Screening

The budding yeast, Saccharomyces cerevisiae, was transformed by the spheroplast method according to the method of Ashby et al. (Ashby, M.N. and Edwards, P.A (1990) J. Biol. Chem. 265, 13157-13164). Namely, HexPS-deficient strain C296-LH3 was transformed with the previously described plasmid library and cultured on leucine-deficient agar plate (leu$^-$ plate) using the top agar method (3% bactoagar, 0.67% yeast nitrogen base, 0.05% yeast extract, 0.05% bacto peptone, 1.0 M sorbitol and 2% glucose).

The transformant having the Leu$^+$ phenotype was inoculated onto YEPG (1% yeast extract, 2% ethanol, 2% bacto peptone and 3% glycerol), D (1% yeast extract, 2% ethanol, 2% bacto peptone, 3% glycerol and 0.1% glucose) and YPD (1% yeast extract, 2% bacto peptone and 2% glucose) agar media followed by incubation for 3 days at 30°C. Clones were selected from the C2960-LH3 transformants with plasmid containing mutated GGPS that grew on the YEPG agar plate, grew and formed colonies larger than those of non-transformed C296-LH3 on the D plate.

This complemented phenotype is considered to indicate that the electron transport chain is active during the respiration reaction, or in other words, that a active coenzyme Q was synthesized in the C296-LH3 cells. Five clones having this complemented phenotype were obtained from 1,400 transformants. As a result of retesting the resulting five clones, not only were they able to grow on YEPG agar plates, but they also possessed the ability to form colonies that were clearly larger than those of YEpG3ΔSpH/C296-LH3, having a plasmid that contains HexPS gene of yeast origin, on D agar plates. The plasmid DNA of these five clones were purified in accordance with routine methods.

These plasmids were named Y-GGPSmut1, y-GGPSmut2, Y-GGPSmut3, Y-GGPSmut4 and Y-GGPSmut5.

Furthermore, since yeast strain C296-LH3 is deficient in HexPS activity, it is unable to biosynthesize coenzyme Q6 which has a hexaprenol group on its side chain. Since coenzyme Q6 is required for non-fermentative metabolism, C296-LH3 forms colonies on media containing a small amount of glucose that are smaller than those of the wild strain, and does not grow on media that only contains a non-fermentative substrate like glycerol for the carbon source. Prior to screening for mutated activity, the effects of expression in wild type GGPS derived from Sulfolobus acidocaldarius were investigated.

On the D plates, strain Y-GGPS/C296-LH3, which is strain C296-LH5 having a plasmid containing the wild type GGPS gene, was found to clearly form colonies smaller than those of YEpG3ΔSpH/C296-LH3 although intermediate to YEpG3ΔSpH/C296-LH3, possessing a plasmid containing HexPS gene of yeast origin, and C296-LH3, not possessing a plasmid. However, Y-GGPS/C296-LH3 was unable to grow on the YEPG plate. This screening method was therefore confirmed to be useful.

Example 4. Determination of DNA Nucleotide Sequence and its Analysis

The nucleotide sequences of DNA coding for the five mutant GGPS contained in the five types of purified plasmids were determined using the Perkin-Elmer Model 373A Fluorescent DNA Sequencer according to the dideoxy chain termination method. Analysis of the nucleotide sequences was performed using the genetic data analysis software, MacMollyTetra.

The amino acid substitution sites as deduced from the nucleotide sequence of each mutant GGPS are shown in Fig. 1. Replacement sites were found at the nucleotide sequence level for all selected mutants. In the case of Mutant 1 which is the Y-GGPSmut1 insertion fragment, replacements were found consisting of mutant methionine at position 85 changing to isoleucine, mutant arginine at position 199 changing to lysine, and mutant aspartic acid at position 312 changing to asparagine. In the case of Mutant 2 which is the Y-GGPSmut2 insertion fragment, the only replacement was mutant phenylalanine at position 118 changing to leucine. In the case of Mutant 3 which is the Y-GGPSmut3 insertion fragment, mutant Phe at position 77 changed to serine, in the case of Mutant 4 which is the Y-GGPSmut4 insertion fragment, mutant phenylalanine at position 77 changed to leucine and mutant valine at position 99 changed to methionine, and in the case of Mutant 5 which is the Y-GGPSmut5 insertion fragment, mutant phenylalanine at position 77 changed to serine and mutant tyrosine at position 101 changed to histidine.

A high proportion of these mutations consist of an aromatic amino acid residue being replaced with a non-aromatic amino acid residue. Phenylalanine at position 77 in particular has the most significant effect on the chain elongation reaction. Phenylalanine at position 77 is located at the five residues upstream from the N-terminal residue of an aspartate-rich domain I. There are two aspartate-rich domain motifs (DDXX(XX)D) that are conserved in prenyl transferase. The diphosphate portion of the substrates are believed to bind here. The amino acid residue located at the fifth position upstream from the N-terminal residue of this aspartate-rich domain, which was focused on for the first time in the present invention, is considered to be extremely important in determining the chain length of the reaction product.

Example 5

A crude extract was prepared from the five selected clones (Y-GGPSmut1/C296-LH3, Y-GGPSmut2/C296-LH3, Y-GGPSmut3/C296-LH3, YGGPSmut4/C296-LH3 and Y-GGPSmut5/C-296-LH3) according to the method of Itoh et al. (Itoh, N. et al. (1984) J Biol. Chem. 259, 13923-13929).

Namely, the above-mentioned yeast was incubated for 4 days at 30°C. Approximately 400 µg of cells were collected by centrifugation and washed once with 800 µl of buffer A (50 mM Tris HCl pH 7.5, 5 mM $MgCl_2$, 50 mM dithiothreitol, 1 M sorbitol). The cells were then suspended in 1.2 mM butter B (50 mM Tris HCl pH 7.5, 5 mM $MgCl_2$, 3 mM dithiothreitol, 1 M sorbitol) followed by the addition of 0.8 mg of zymolyase and incubation for 1 hour at 30°C.

The prepared spheroblasts were washed three times with buffer B and suspended in 1 ml of buffer C (50 mM Tris HCl pH 7.0, 10 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA). Ultrasonic treatment was performed 10 times on the suspension in ice at two minute intervals, performing treatment for 10 seconds at a time at maximum output using a Branson Sonifier. The lysates were incubated for 1 hour at 55°C, and after inactivating prenyl transferase(s) of the host cells, the lysates were centrifuged for 10 minutes at 10,000 x g. The resulting supernatant was used as a mutant GGPS crude enzyme solution and assay of prenyl transferase activity.

The results of performing an assay of prenyl transferase activity by LKC-18 thin layer chromatography using this mutant GGPS crude enzyme liquid prepared from yeast are shown in Figs. 2 and 3.

After carrying out the enzyme reaction at 55°C, polyprenyl diphosphate was extracted with 1-butanol after which the 1-butanol was evaporated with a nitrogen gas flow. The resulting polyprenyl diphosphate was treated with acid phosphatase in accordance with the method of Fujii et al. (Fujii et al. (1982) Biochim. Biophys. Acta. 712, 716-718). The hydrolysis product was extracted with pentane and after performing thin layer chromatography using acetone/H_20 (9:1) for the developing solution, the distribution of radioactivity was analyzed with the Fuji Film Model BAS2000 Bio-image Analyzer. The alcohols as the authentic standards, on which thin layer chromatography was performed simultaneously, followed by staining with iodine vapor (geranyol, farnesol, geranylgeraniol), were used to determine the developing locations.

Fig. 2 shows the result of reacting using [1-^{14}C]IPP and (all-E)-FPP for the substrates, while Fig. 3 shows the result of reacting using [1-^{14}C]IPP and (all-E)-GGPP for the substrates. Spots a through c correspond to the authentic standard samples, a indicating geraniol, b indicating (all-E)-farnesol, and c indicating (all-E)-geranylgeranyol. Ori indicates the sample-stopping point, S.F. indicates the solvent front.

On the basis of these results, in the case of using GGPP for the allylic substrate, it was shown that each mutant GGPS is able to synthesize geranylfarnesyl diphosphate (GFPP) that is one isoprene unit longer than the reaction product of the wild type enzyme. On the other hand, the wild type GGPS is unable to synthesize the reaction product same as or longer than the chain length of GFPP at a level that allows detection. In the case of using FPP for the allylic substrate, the product ratio of GGPP/GFPP indicated by the mutant GGPS was different from each other.

Example 6. Preparation of Mutant GGPS from E. coli

In order to ensure that the analysis is performed more accurately, each mutant GGPS was over expressed in E. coli strain of XL 1-Blue. Namely, each of the five plasmids Y-GGPSmut1, Y-GGPSmut2, Y-GGPSmut3, Y-GGPSmut4 and Y-GGPSmut5 obtained in screening was digested with HindIII to obtain HindIII DNA fragments that code tor the mutant GGPS. These HindIII DNA fragments were inserted at the HindIII site of the plasmid vector pBluescript (KS(+)) to obtain pBS-GGPSmut1, pBS-GGPSmut2, pBS-GGPSmut3, pBS-GGPSmut4 and pBS-GGPSmut5 respectively.

E. coli XL1-Blue was transformed with pBS-GGPSmut1, pBS-GGPSmut2, pBS-GGPSmut3, pBS-GGPSmut4 and pBS-GGPSmut5 and cultured according to the method described in Molecular Cloning (Sambrook, J. et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). After collecting the bacterial cells, the bacterial cells were ultrasonically homogenized in 50 mM Tris HCl buffer containing 10 mM 2-mercaptoethanol and 1 mM EDTA. After heat treating the homogenate for 1 hour at 55°C, it was centrifuged for 10 minutes at 100,000 x g. The supernatant was then collected as the crude enzyme solution which was assayed for PTase activity.

Assay was performed by analysis of product with LKC-18 thin layer chromatography and by determination of enzyme activity. For thin layer chromatography, DMAPP, GPP, (all-E)-FPP and (all-E)-GGPP were used for the allylic substrates, and after reacting in the same manner as Example 5, LKC thin layer chromatography was performed in the same manner as Example 5. Those results are shown in Figs. 4 and 5.

Fig. 4(A) is the result of reacting [1-^{14}C]IPP with DMAPP for the substrate, and (B) is the result of reacting [1-^{14}C]IPP with GPP for the substrate. Fig. 5(C) is a result of reacting [1-^{14}C]IPP with (all-E)-FPP for the substrate, while (D) is a result of reacting [1-^{14}C]IPP with (all-E)-GGPP for the substrate. Ellipses a through c show the positions of the authentic standard samples, a indicating geraniol, b indicating (all-E)-farnesol and c indicating (all-E)-geranylgeraniol. Ori indicates the sample-spoting point, while S.F. indicates the solvent front.

The prenyl transferase activity was assaied as follows. 1 ml of assay mixture, containing 25 nmol of [1-^{14}C]IPP (37

GBq/mol), 25 nmol of allylic substrate (DMAPP, GPP, (all-E)-FPP or (all-E)-GGPP), 5 μmol of $MgCl_2$, 10 μmol of phosphate buffer (pH 5.8) and the enzyme solution, was incubated for 60 minutes at 55°C.

The reaction was stopped by cooling rapidly on ice. After adding 3.5 ml of water-saturated 1-butanol to the chilled mixture and shaking vigorously, the 1-butanol layer was washed with NaCl-saturated water and ^{14}C radioactivity was measured with a liquid scintillation counter. 1 unit of enzyme activity was defined as the amount for which 1 nmol of [1-^{14}C]IPP is incorporated into elongated prenyl diphosphate (polyprenyl diphosphate) that can be extracted with the 1-butanol layer. Those results are shown in the Table.

Table

Substrate	Relative Activity (dpm)	Product Distribution				
		GPP	FPP	GGPP	GFPP	FFPP
		%	%	%	%	%
Mutant 1						
DMAPP	31,800	23.2	8.77	29.6	38.0	0.45
GPP	5,260	nd*	38.8	30.9	30.4	0.02
FPP	4,340	nd*	nd*	65.1	35.0	nd*
GGPP	998	nd*	nd*	nd*	100	nd*
Mutant 2						
DMAPP	15,800	1.44	0.66	89.0	8.86	nd*
GPP	7,050	nd*	20.3	74.9	4.89	nd*
FPP	6,080	nd*	nd*	89.5	10.5	nd*
GGPP	379	nd*	nd*	nd*	100	nd*
Mutant 3						
DMAPP	24,900	3.40	27.4	16.6	51.6	0.92
GPP	9,890	nd*	64.7	9.37	24.5	1.44
FPP	7,280	nd*	nd*	30.4	69.6	nd*
GGPP	3,200	nd*	nd*	nd*	100	nd*
Mutant 4						
DMAPP	16,700	4.93	4.07	73.2	17.8	nd*
GPP	7,460	nd*	38.4	51.3	10.3	nd*
FPP	5,650	nd*	nd*	85.9	14.1	nd*
GGPP	551	nd*	nd*	nd*	100	nd*
Mutant 5						
DMAPP	23,600	27.1	18.6	12.8	40.4	1.12
GPP	9,070	nd*	59.3	13.0	26.1	1.56
FPP	8,960	nd*	nd*	32.0	68.0	nd*
GGPP	2,200	nd*	nd*	nd*	100	nd*
Wild type						
DMAPP	13,600	5.61	0.43	94.0	nd*	nd*
GPP	6,640	nd*	17.2	82.8	nd*	nd*
FPP	4,650	nd*	nd*	100	nd*	nd*
GGPP	nd*	nd*	nd*	nd*	nd*	nd*
nd: Not detected						

Each mutant GGPS exhibited activity that synthesizes polyprenyl diphosphate having a longer chain length than GGPP. The wild type GGPS as well as each mutant GGPS reacted the best with DMAPP amongst the four allylic substrates. In addition, relative activity when allylic substrates were used that had a shorter chain length than FPP exhibited similar values. However, relative activity and product distribution when GGPP was used for the allylic substrate were considerably different.

When DMAPP, GPP and FPP were used for the allylic substrates, Mutant 1, which is coded for by the insert DNA of plasmid pBS-GGPSmut1, yielded the major reaction products of GFPP and GGPP. In particular, when DMAPP was used for the allylic substrate, only a slight amount of hexaprenyl diphosphate (HexPP) was detected as the reaction product. Although the distribution of reaction products varied between each allylic substrate, the proportion of product produced in one cycle of the condensation reaction was large.

In the case of Mutant 2 coded for by the insert DNA of plasmid pBS-GGPSmut2, the major product was GGPP and the proportion of GFPP was roughly 10%. HexPP was not detected.

Mutant 3, which is coded for by the insert DNA of plasmid pBS-GGPSmut3, and Mutant 5, which is coded for by the insert DNA of plasmid pBS-GGPSmut5, demonstrated similar characteristics. These mutants exhibited strong GFPP synthesis activity, while also synthesizing a small amount of HexPP.

Mutant 4, which is coded for by the insert DNA of plasmid pBS-GGPSmut4, yielded GGPP as the major product, while the proportion of GFPP was roughly 15%. FPP was effectively synthesized when GPP was used for the allylic substrate..

SEQUENCE LISTING

SEQ ID NO: 1

Sequence Length: 993

Sequence Type: Nucleic acid

Strandness: Double strand

Topology: Linear

Molecular Type: Genomic DNA

Source

Organism: Sulfolobus acidocaldarius

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Sequence Length: 993
Sequence Type: Nucleic acid
Strandness: Double strand
Topology: Linear
Molecular Type: Mutated genomic DNA
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Sequence Length: 993
Sequence Type: Nucleic acid
Strandness: Double strand
Topology: Linear
Molecular Type: Mutated genomic DNA
Sequence

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His Asp Asp Ile Met Asp Gln Asp Asn Ile Arg Arg Gly Leu Pro Thr
85 90 95

GTC CAC GTG AAA TAC GGC TTA CCC TTA GCA ATA TTA GCT GGG GAT TTA 336
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Asn Gln Val Ser Ser Lys Ser Asp Ile Pro Gly Lys Ala Leu Lys Tyr
305                 310                 315                 320
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CTA GCT GAA TTT ACG ATA AGA AGG AGA AAA TAA 993
Leu Ala Glu Phe Thr Ile Arg Arg Arg Lys
325 330

SEQ ID NO: 4
Sequence Length: 993
Sequence Type: Nucleic acid
Strandness: Double strand
Topology: Linear
Molecular Type: Mutated genomic DNA
Sequence

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35 40 45
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His Asp Asp Ile Met Asp Gln Asp Asn Ile Arg Arg Gly Leu Pro Thr
85 90 95
GTC CAC GTG AAA TAC GGC TTA CCC TTA GCA ATA TTA GCT GGG GAT TTA 336
Val His Val Lys Tyr Gly Leu Pro Leu Ala Ile Leu Ala Gly Asp Leu
100 105 110
CTA CAT GCA AAG GCT TTT CAG CTC TTA ACC CAG GCT CTT AGA GGT TTG 384
Leu His Ala Lys Ala Phe Gln Leu Leu Thr Gln Ala Leu Arg Gly Leu
115 120 125

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Pro Ser Glu Thr Ile Ile Lys Ala Phe Asp Ile Phe Thr Arg Ser Ile
    130                 135                 140

ATA ATT ATA TCC GAA GGA CAG GCA GTA GAT ATG GAA TTT GAG GAC AGA      480
Ile Ile Ile Ser Glu Gly Gln Ala Val Asp Met Glu Phe Glu Asp Arg
145                 150                 155                 160

ATT GAT ATA AAG GAG CAG GAA TAC CTT GAC ATG ATC TCA CGT AAG ACA      528
Ile Asp Ile Lys Glu Gln Glu Tyr Leu Asp Met Ile Ser Arg Lys Thr
                165                 170                 175

GCT GCA TTA TTC TCG GCA TCC TCA ACT ATA GGC GCA CTT ATT GCT GGT      576
Ala Ala Leu Phe Ser Ala Ser Ser Ser Ile Gly Ala Leu Ile Ala Gly
            180                 185                 190

GCT AAT GAT AAT GAT GTA AGA CTG ATG TCT GAT TTC GGT ACG AAT CTA      624
Ala Asn Asp Asn Asp Val Arg Leu Met Ser Asp Phe Gly Thr Asn Leu
        195                 200                 205

GGT ATT GCA TTT CAG ATT GTT GAC GAT ATC TTA GGT CTA ACA GCA GAC      672
Gly Ile Ala Phe Gln Ile Val Asp Asp Ile Leu Gly Leu Thr Ala Asp
    210                 215                 220

GAA AAG GAA CTT GGA AAG CCT GTT TTT AGT GAT ATT AGG GAG GGT AAA      720
Glu Lys Glu Leu Gly Lys Pro Val Phe Ser Asp Ile Arg Glu Gly Lys
225                 230                 235                 240

AAG ACT ATA CTT GTA ATA AAA ACA CTG GAG CTT TGT AAA GAG GAC GAG      768
Lys Thr Ile Leu Val Ile Lys Thr Leu Glu Leu Cys Lys Glu Asp Glu
                245                 250                 255

AAG AAG ATT GTC CTA AAG GCG TTA GGT AAT AAG TCA GCC TCA AAA GAA      816
Lys Lys Ile Val Leu Lys Ala Leu Gly Asn Lys Ser Ala Ser Lys Glu
            260                 265                 270

GAA TTA ATG AGC TCA GCA GAT ATA ATT AAG AAA TAC TCT TTA GAT TAT      864
Glu Leu Met Ser Ser Ala Asp Ile Ile Lys Lys Tyr Ser Leu Asp Tyr
        275                 280                 285

GCA TAC AAT TTA GCA GAG AAA TAT TAT AAA AAT GCT ATA GAC TCT TTA      912
Ala Tyr Asn Leu Ala Glu Lys Tyr Tyr Lys Asn Ala Ile Asp Ser Leu
    290                 295                 300

AAT CAA GTC TCC TCT AAG AGT GAT ATA CCT GGA AAG GCT TTA AAA TAT      960
Asn Gln Val Ser Ser Lys Ser Asp Ile Pro Gly Lys Ala Leu Lys Tyr
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```

CTA GCT GAA TTT ACG ATA AGA AGG AGA AAA TAA 993
Leu Ala Glu Phe Thr Ile Arg Arg Arg Lys TER
325 330

SEQ ID NO: 5
Sequence Length: 993
Sequence Type: Nucleic acid
Strandness: Double strand
Topology: Linear
Molecular Type: Mutated genomic DNA
Sequence

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5 10 15

CAC ATT ATT AAG AGC TAT ATA TCT GGA GAT GTT CCT AAA CTA TAT GAA 96
Asp Ile Ile Lys Ser Tyr Ile Ser Gly Asp Val Pro Lys Leu Tyr Glu
20 25 30

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Ala Ser Tyr His Leu Phe Thr Ser Gly Gly Lys Arg Leu Arg Pro Leu
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50 55 60

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65 70 75 80

CAT GAT GAT ATT ATG GAT CAA GAT AAT ATC AGA AGA CGG TTA CCC ACA 288
His Asp Asp Ile Met Asp Gln Asp Asn Ile Arg Arg Gly Leu Pro Thr
85 90 95

GTC CAC ATG AAA TAC GGC TTA CCC TTA GCA ATA TTA GCT GGG GAT TTA 336
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100 105 110

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115 120 125

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ATA ATT ATA TCC GAA GGA CAG GCA GTA GAT ATG GAA TTT GAG GAC AGA    480
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145                 150                 155                 160
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Ala Asn Asp Asn Asp Val Arg Leu Met Ser Asp Phe Gly Thr Asn Leu
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GGT ATT GCA TTT CAG ATT GTT GAC GAT ATC TTA GGT CTA ACA GCA GAC    672
Gly Ile Ala Phe Gln Ile Val Asp Asp Ile Leu Gly Leu Thr Ala Asp
    210                 215                 220
GAA AAG GAA CTT GGA AAG CCT GTT TTT AGT GAT ATT AGG GAG GGT AAA    720
Glu Lys Glu Leu Gly Lys Pro Val Phe Ser Asp Ile Arg Glu Gly Lys
225                 230                 235                 240
AAG ACT ATA CTT GTA ATA AAA ACA CTG GAG CTT TGT AAA GAG GAC GAG    768
Lys Thr Ile Leu Val Ile Lys Thr Leu Glu Leu Cys Lys Glu Asp Glu
                245                 250                 255
AAG AAG ATT GTC CTA AAG GCG TTA GGT AAT AAG TCA GCC TCA AAA GAA    816
Lys Lys Ile Val Leu Lys Ala Leu Gly Asn Lys Ser Ala Ser Lys Glu
            260                 265                 270
GAA TTA ATG AGC TCA GCA GAT ATA ATT AAG AAA TAC TCT TTA GAT TAT    864
Glu Leu Met Ser Ser Ala Asp Ile Ile Lys Lys Tyr Ser Leu Asp Tyr
        275                 280                 285
GCA TAC AAT TTA GCA GAG AAA TAT TAT AAA AAT GCT ATA GAC TCT TTA    912
Ala Tyr Asn Leu Ala Glu Lys Tyr Tyr Lys Asn Ala Ile Asp Ser Leu
    290                 295                 300
AAT CAA GTC TCC TCT AAG AGT GAT ATA CCT GGA AAG GCT TTA AAA TAT    960
Asn Gln Val Ser Ser Lys Ser Asp Ile Pro Gly Lys Ala Leu Lys Tyr
305                 310                 315                 320
```

CTA GCT GAA TTT ACG ATA AGA AGG AGA AAA TAA 993
Leu Ala Glu Phe Thr Ile Arg Arg Arg Lys TER
325 330

SEQ ID NO: 6
Sequence Length: 993
Sequence Type: Nucleic acid
Strandness: Double strand
Topology: Linear
Molecular Type: Mutated genomic DNA
Sequence

ATG AGT TAC TTT GAC AAC TAT TTT AAT GAG ATT GTT AAT TCT GTA AAC 48
Met Ser Tyr Phe Asp Asn Tyr Phe Asn Glu Ile Val Asn Ser Val Asn
5 10 15
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Asp Ile Ile Lys Ser Tyr Ile Ser Gly Asp Val Pro Lys Leu Tyr Glu
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GCC TCA TAT CAT TTG TTT ACA TCT GGA GGT AAG AGG TTA AGA CCA TTA 144
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His Asp Asp Ile Met Asp Gln Asp Asn Ile Arg Arg Gly Leu Pro Thr
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GTC CAC GTG AAA CAC GGC TTA CCC TTA GCA ATA TTA GCT GGG GAT TTA 336
Val His Val Lys His Gly Leu Pro Leu Ala Ile Leu Ala Gly Asp Leu
100 105 110
CTA CAT GCA AAG GCT TTT CAG CTC TTA ACC CAG GCT CTT AGA GGT TTG 384
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115 120 125

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CCA AGT GAA ACC ATA ATT AAG GCT TTC GAT ATT TTC ACT CGT TCA ATA      432
Pro Ser Glu Thr Ile Ile Lys Ala Phe Asp Ile Phe Thr Arg Ser Ile
    130                 135                 140
ATA ATT ATA TCC GAA GGA CAG GCA GTA GAT ATG GAA TTT GAG GAC AGA      480
Ile Ile Ile Ser Glu Gly Gln Ala Val Asp Met Glu Phe Glu Asp Arg
145                 150                 155                 160
ATT GAT ATA AAG GAG CAG GAA TAC CTT GAC ATG ATC TCA CGT AAG ACA      528
Ile Asp Ile Lys Glu Gln Glu Tyr Leu Asp Met Ile Ser Arg Lys Thr
                165                 170                 175
GCT GCA TTA TTC TCG GCA TCC TCA AGT ATA GGC GCA CTT ATT GCT GGT      576
Ala Ala Leu Phe Ser Ala Ser Ser Ser Ile Gly Ala Leu Ile Ala Gly
            180                 185                 190
GCT AAT GAT AAT GAT GTA AGA CTG ATG TCT GAT TTC GGT ACG AAT CTA      624
Ala Asn Asp Asn Asp Val Arg Leu Met Ser Asp Phe Gly Thr Asn Leu
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GGT ATT GCA TTT CAG ATT GTT GAC GAT ATC TTA GGT CTA ACA GCA GAC      672
Gly Ile Ala Phe Gln Ile Val Asp Asp Ile Leu Gly Leu Thr Ala Asp
    210                 215                 220
GAA AAG GAA CTT GGA AAG CCT GTT TTT AGT GAT ATT AGG GAG GGT AAA      720
Glu Lys Glu Leu Gly Lys Pro Val Phe Ser Asp Ile Arg Glu Gly Lys
225                 230                 235                 240
AAG ACT ATA CTT GTA ATA AAA ACA CTG GAG CTT TGT AAA GAG GAC GAG      768
Lys Thr Ile Leu Val Ile Lys Thr Leu Glu Leu Cys Lys Glu Asp Glu
                245                 250                 255
AAG AAG ATT GTC CTA AAG GCG TTA GGT AAT AAG TCA GCC TCA AAA GAA      816
Lys Lys Ile Val Leu Lys Ala Leu Gly Asn Lys Ser Ala Ser Lys Glu
            260                 265                 270
GAA TTA ATG AGC TCA GCA GAT ATA ATT AAG AAA TAC TCT TTA GAT TAT      864
Glu Leu Met Ser Ser Ala Asp Ile Ile Lys Lys Tyr Ser Leu Asp Tyr
        275                 280                 285
GCA TAC AAT TTA GCA GAG AAA TAT TAT AAA AAT GCT ATA GAC TCT TTA      912
Ala Tyr Asn Leu Ala Glu Lys Tyr Tyr Lys Asn Ala Ile Asp Ser Leu
    290                 295                 300
AAT CAA GTC TCC TCT AAG AGT GAT ATA CCT GGA AAG GCT TTA AAA TAT      960
Asn Gln Val Ser Ser Lys Ser Asp Ile Pro Gly Lys Ala Leu Lys Tyr
305                 310                 315                 320
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CTA GCT GAA TTT ACG ATA AGA AGG AGA AAA TAA 993
Leu Ala Glu Phe Thr Ile Arg Arg Arg Lys TER
325 330

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Strandness: Single strand
Topology: Linear
Molecular Type: Synthetic DNA
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SEQ ID NO: 9
Sequence Length: 28
Sequence Type: Nucleic acid
Strandness: Single strand
Topology: Linear
Molecular Type: Synthetic DNA
Sequence
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The present invention discloses a mutated enzyme comprising a geranylgeranil diphosphate synthase having its origin in wild type Sulfolobus acidocaldarius wherein, one of at least phenylalanine at position 77, methionine at position 85, valine at position 99, tyrosine at position 101, phenylalanine at position 118, arginine at position 199 and aspartic acid at position 312 is substituted with another amino acid.

Claims

1. A mutated enzyme wherein at least one of phenylalanine at position 77, methionine at position 85, valine at position 99, tyrosine at position 101, phenylalanine at position 118, arginine at position 199 and aspartic acid at position 312

in a geranylgeranil diphosphate synthase of Sulfolobus acidocaldarius origin, is replaced with another amino acid, which enzyme is able to form prenyl diphosphate having at least 25 carbon atoms, or a modified mutant enzyme that is modified by replacing, deleting and/or adding one to several amino acids, which enzyme maintains the activity of the above-mentioned enzyme.

2. An enzyme as set forth in claim 1 wherein at least phenylalanine at position 77 is substituted with another amino acid,

3. An enzyme as set forth in claim 2 wherein said amino acid is a non-aromatic amino acid.

4. An enzyme as set forth in claim 2 wherein phenylalanine at position 77 is substituted with a non-aromatic amino acid.

5. An enzyme as set forth in either claim 2 or claim 3 wherein valine at position 99 is further substituted by another amino acid.

6. An enzyme as set forth in either claim 2 or claim 3 wherein tyrosine at position 101 is further substituted by another amino acid.

7. An enzyme as set forth in claim 1 wherein at least methionine at position 85, arginine at position 199, and aspartic acid at position 312 are substituted with other amino acids.

8. An enzyme as set forth in claim 1 wherein at least phenylalanine at position 118 is substituted with another amino acid.

9. A gene that codes for an enzyme as set forth in any of claims 1 through 8.

10. An expression vector that contains a gene as set forth in claim 9.

11. A host transfected by an expression vector as set forth in claim 10.

12. A process for production of an enzyme according to claim 11 in a process for producing an enzyme as set forth in any of claims 1 through 8. Claim 1, comprising the steps of

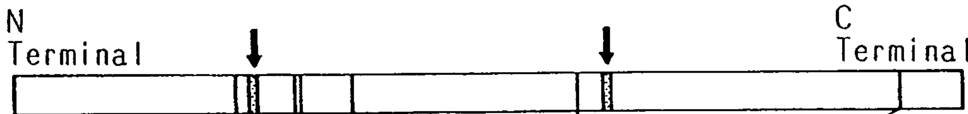
culturing host cells transformed with an expression vector comprising a gene coding for the enzyme of claim 1, and
recovering the enzyme.

13. A process for production of a mutated prenyl diphosphate synthase comprising the step of:

culturing host cells transformed with a gene mutated by substitution of a codon for the amino acid residue at fine upstream to the amino terminal of the aspartic acid-rich domain 1 with a codon for a non-aromatic amino acid residue so as to express the mutated prenyl diphosphate synthase which can produce longer chain of prenyl diphosphate than those produced by the original wild-type prenyl diphosphate synthase.

14. A process for production of a prenyl diphosphate equal to or larger than those having 25 carbon atoms, comprising reacting an enzyme according to any one of claims 1 to 8 or an enzyme produced by a process according to claim 12 or 13 with a substrate selected from isopentenyl diphosphate, dimethylallyl diphosphate, geranyl diphosphate, farnesil diphosphate and geranylgeranyl diphosphate.

Fig.1



77	85	99	101	118	199	312	
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				L			mutant 2
S							mutant 3
L		M					mutant 4
S			H				mutant 5

Fig. 2

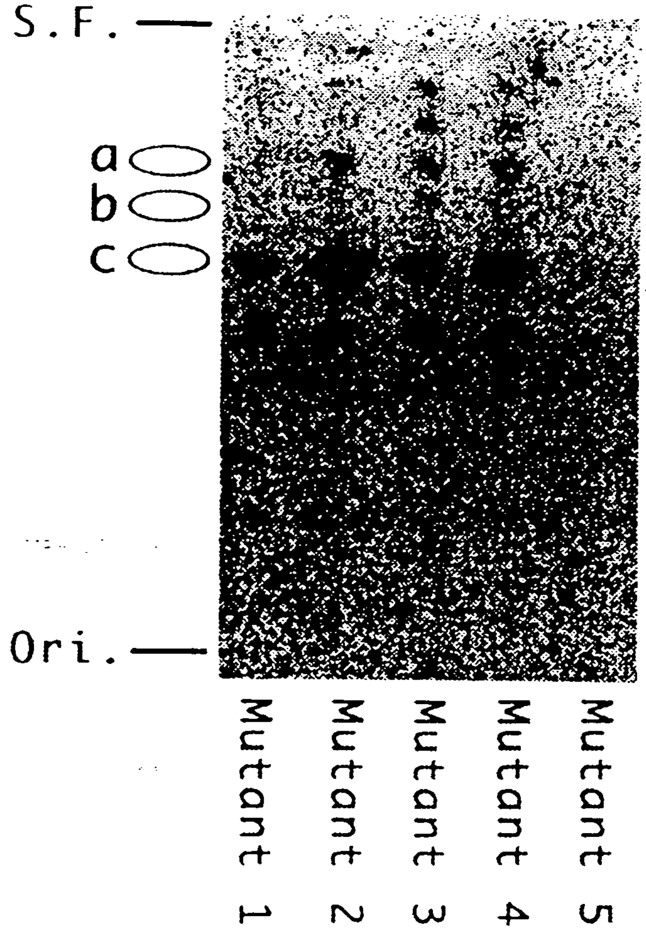
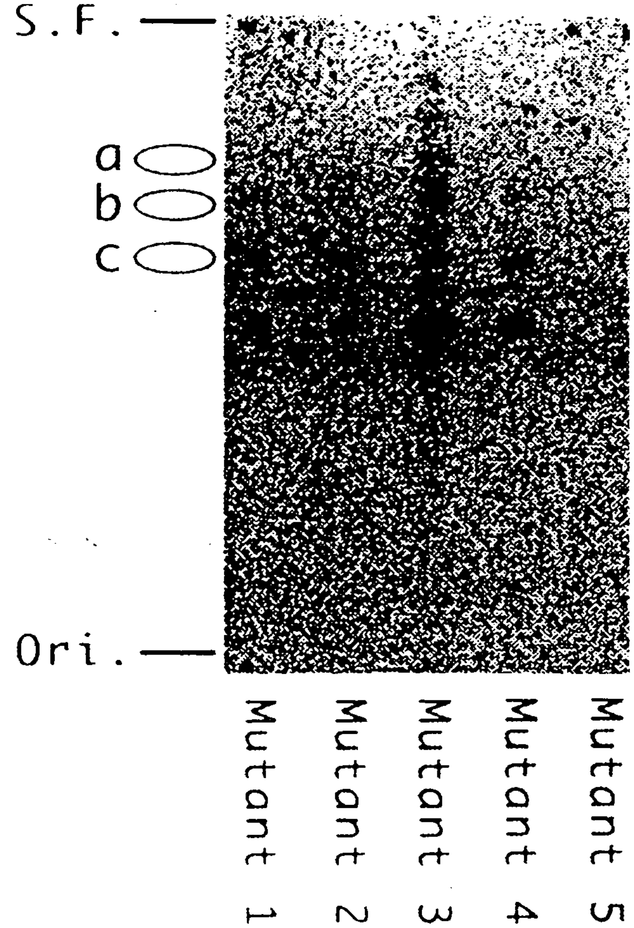


Fig.3



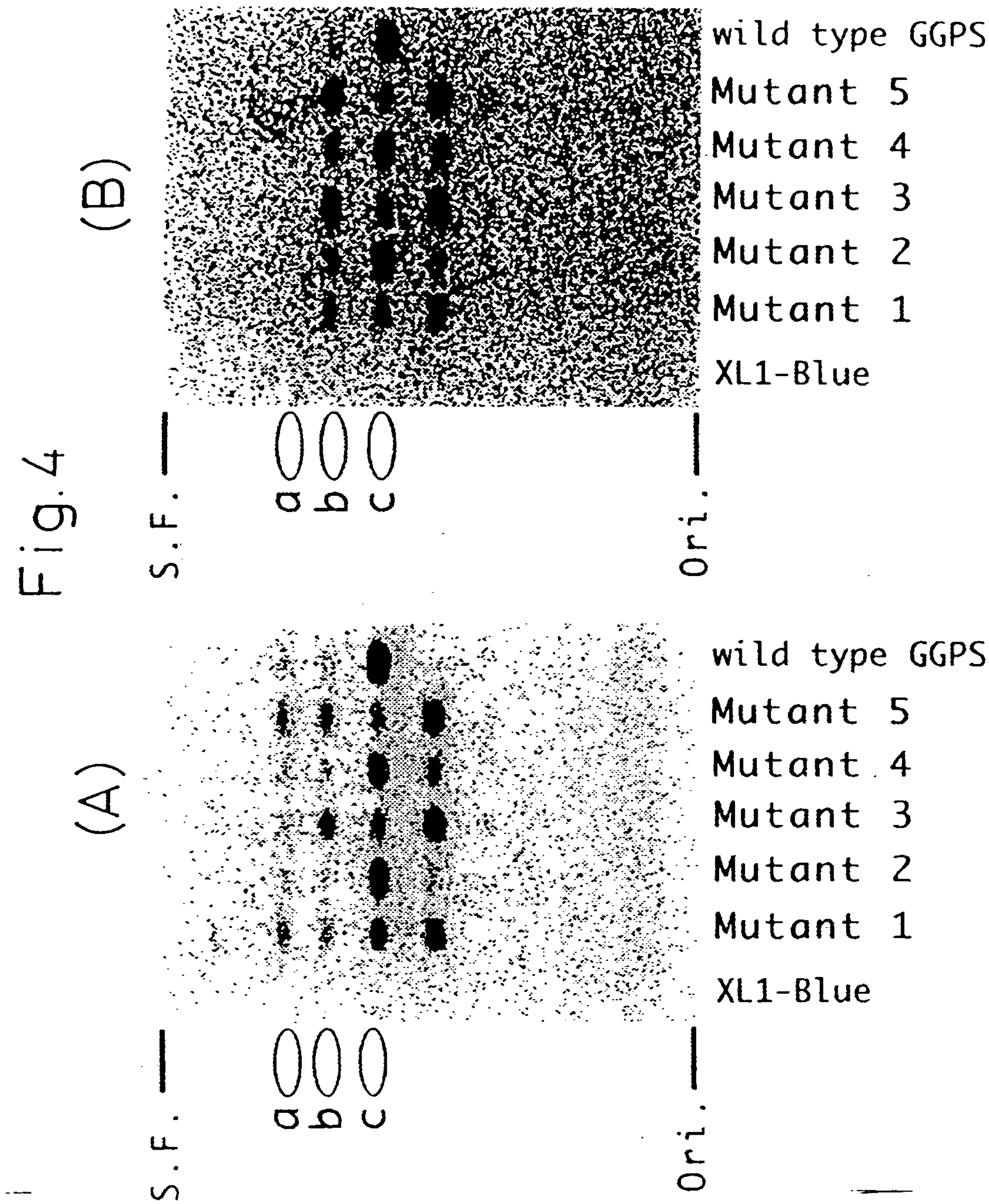


Fig.4
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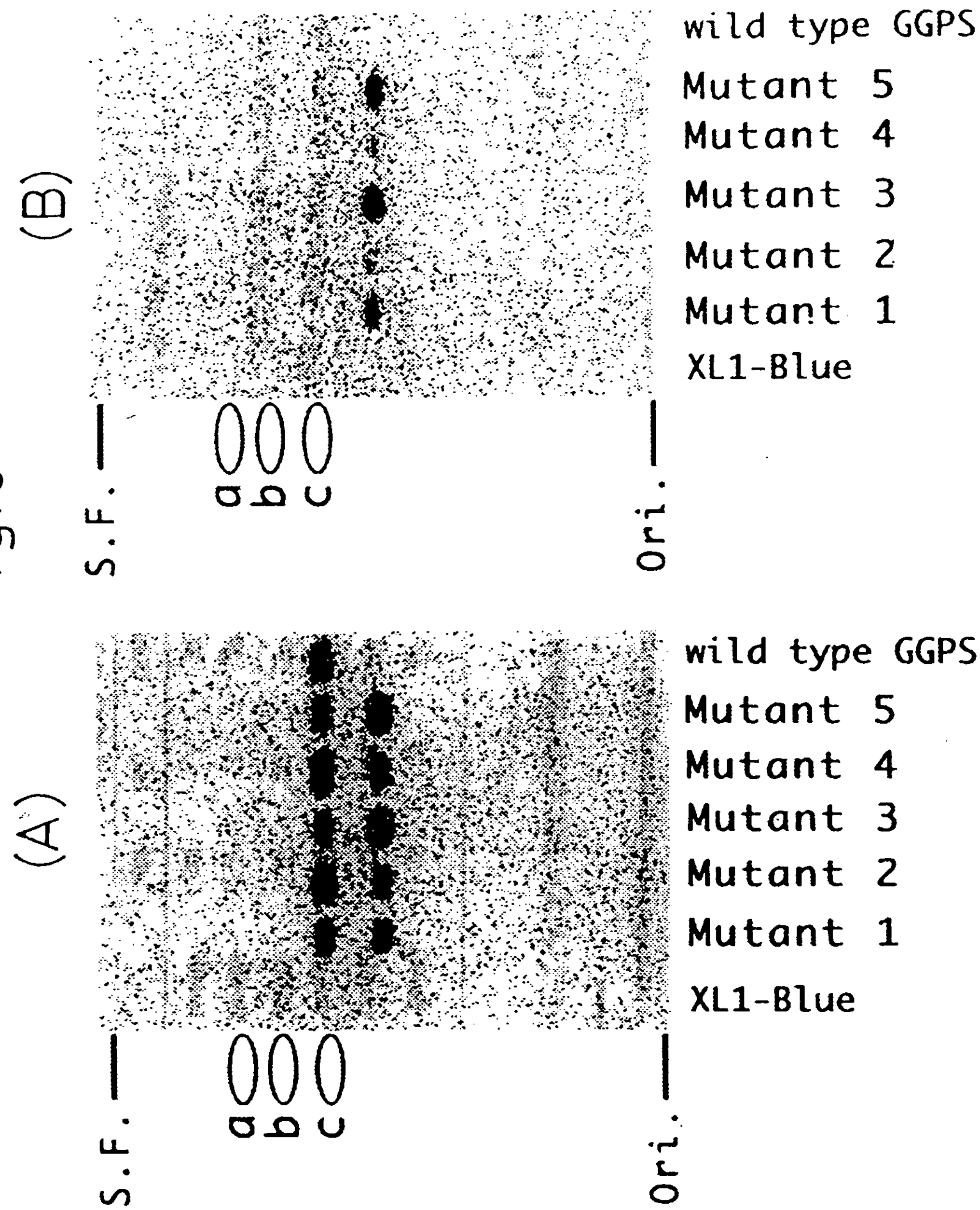
Mutant 4
Mutant 3
Mutant 2
Mutant 1
XL1-Blue
S.F.
a
b
c
Ori.
(A)
wild type GGPS
Mutant 5
Mutant 4
Mutant 3
Mutant 2
Mutant 1
XL1-Blue
S.F.
a
b
c
Ori.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wild type GGPS
Mutant 5
Mutant 4
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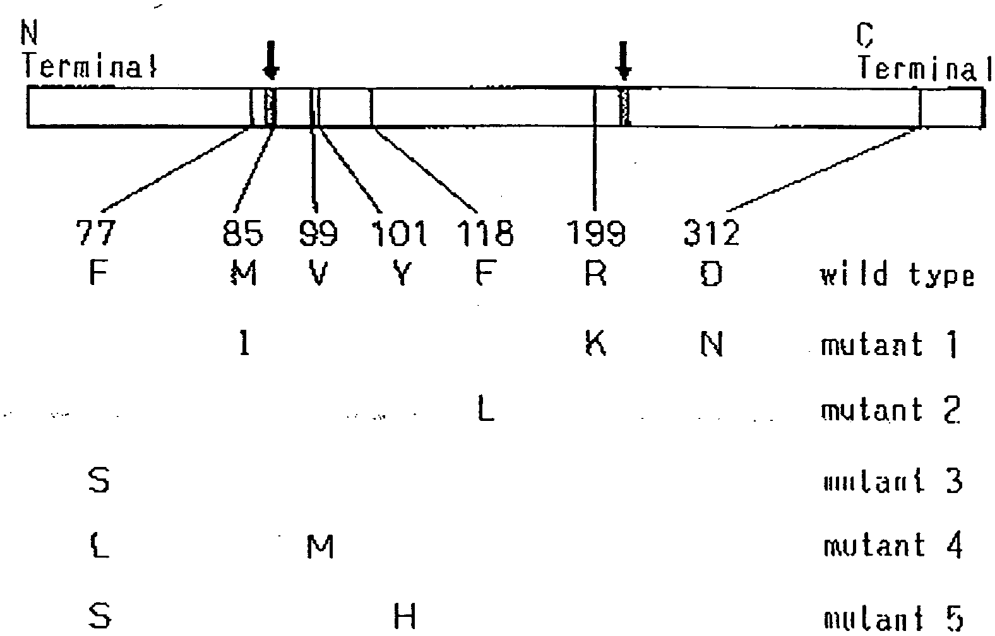
XL1-Blue
S.F.
a
b
c
Ori.
(A)
wild type GGPS
Mutant 5
Mutant 4
Mutant 3
Mutant 2
Mutant 1
XL1-Blue
S.F.
a
b
c
Ori.

Fig.5

Fig. 1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C Terminal
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L mutant 2
S mutant 3
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S H mutant 5

Fig. 2

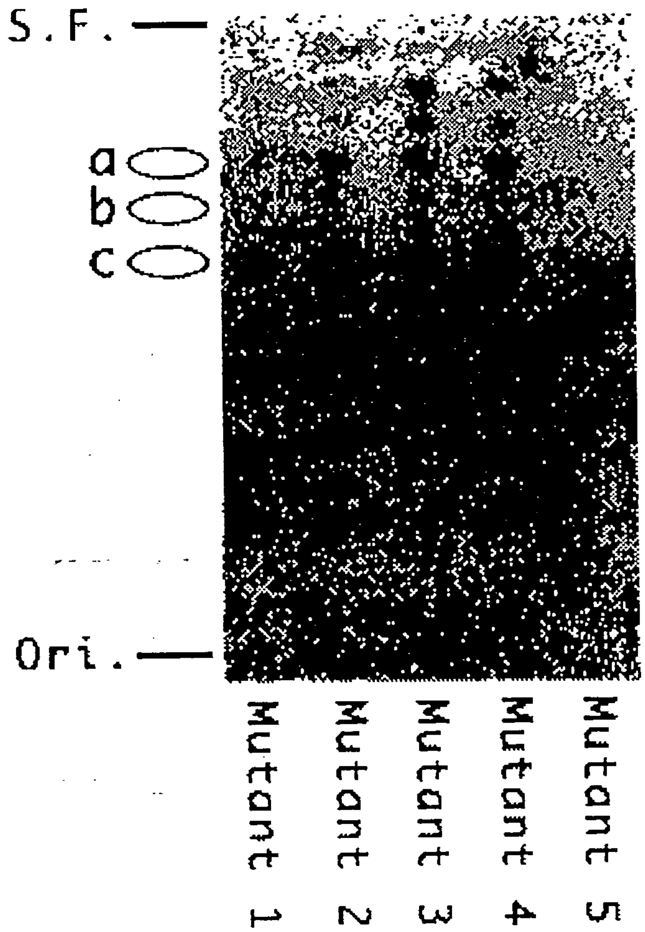


Fig.3

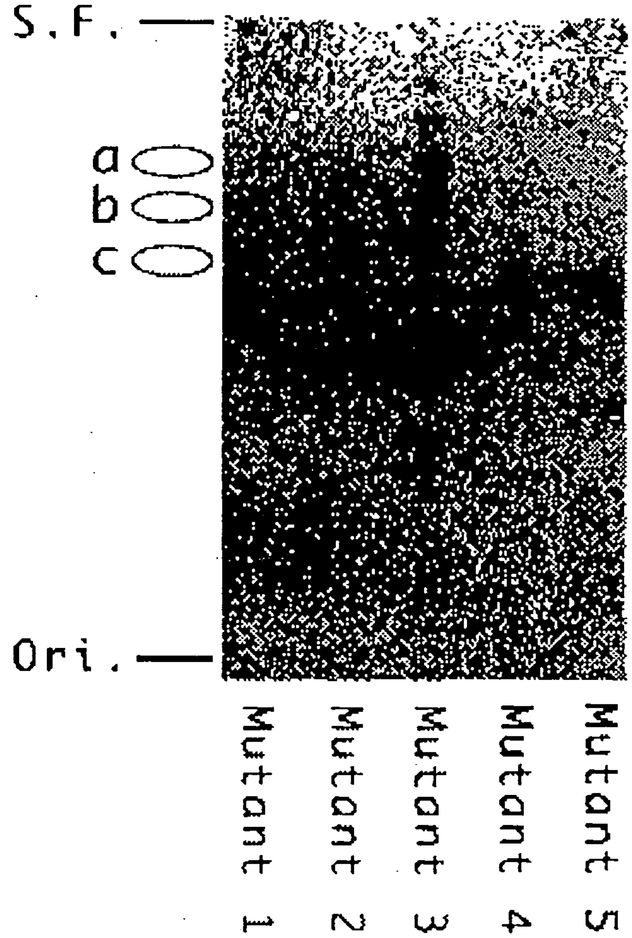
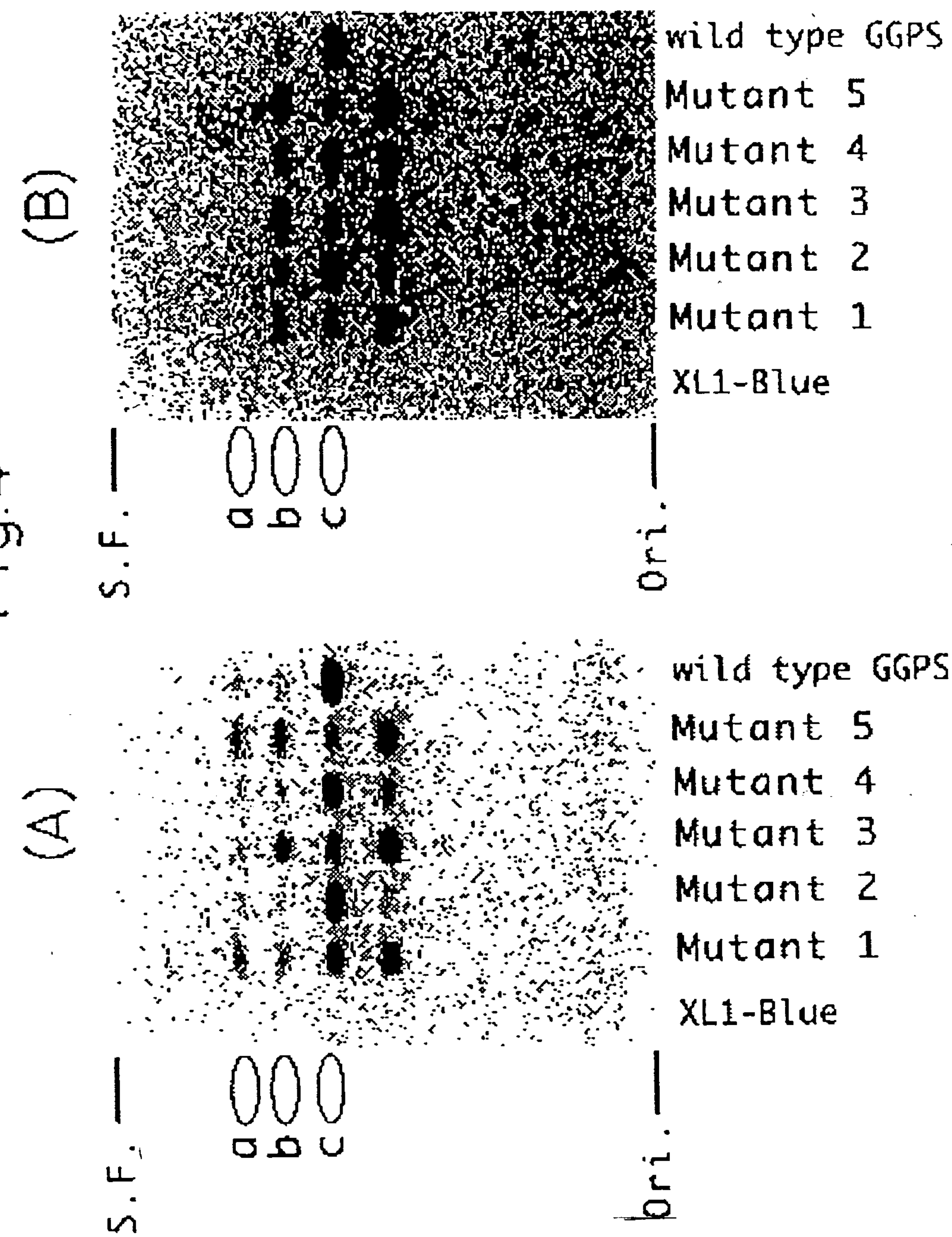


Fig. 4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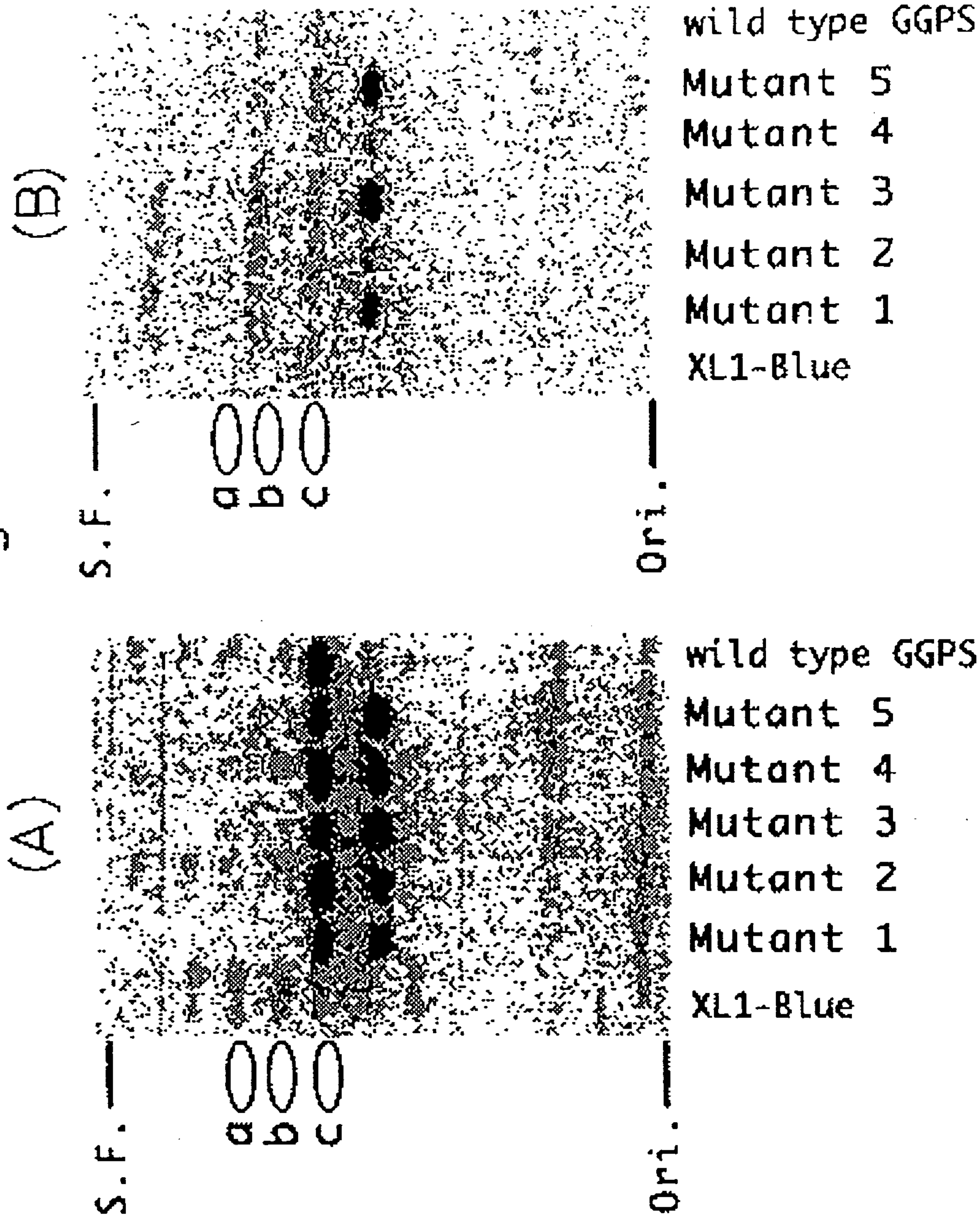
wild type GGPS
Mutant 5
Mutant 4
Mutant 3
Mutant 2
Mutant 1
XL1-Blue
S.F.
a
b
c
Ori.
(A)
wild type GGPS
Mutant 5
Mutant 4
Mutant 3
Mutant 2
Mutant 1
XL1-Blue
S.F.
a
b
c
Ori.

Fig.5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Mutant 5
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(A)
wild type GGPS
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Mutant 4
Mutant 3
Mutant 2
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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

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(21) Application number: 96113930.0

(22) Date of filing: 30.08.1996

(51) Int. Cl.6: **C12N 9/10**, C12N 15/31, C12N 15/54, C12N 15/63, C12N 1/21, C07K 14/195

(84) Designated Contracting States:
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(30) Priority: 01.09.1995 JP 247043/95

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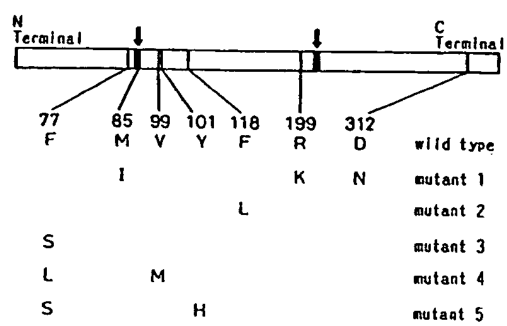
Toyota-shi, Aichi-ken, 471 (JP)
- Nishino, Tokuzo
Toyota-shi, Aichi-ken, 471 (JP)
- Hirooka, Kazutake
Toyota-shi, Aichi-ken (JP)
- Hemmi, Hisashi
Toyota-shi, Aichi-ken (JP)

(74) Representative: Tiedtke, Harro, Dipl.-Ing.
Patentanwaltsbüro
Tiedtke-Bühling-Kinne & Partner
Bavariaring 4
80336 München (DE)

(54) **Long-chain prenyl diphosphate synthase**

(57) The present invention discloses a mutated enzyme comprising a geranylgeranil diphosphate synthase having its origin in wild type Sulfolobus acidocaldarius wherein, one of at least phenylalanine at position 77, methionine at position 85, valine at position 99, tyrosine at position 101, phenylalanine at position 118, arginine at position 199 and aspartic acid at position 312 is substituted with another amino acid.

Fig. 1



EP 0 763 542 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 11 3930

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 31, 1996, pages 18831-18837, XP002031065 SHIN-ICHI OHNUMA ET AL.: "Convertion of product specificity of archaebacterial geranylgeranyl-diphosphate synthase" *see the whole article* ---	1-14	C12N9/10 C12N15/31 C12N15/54 C12N15/63 C12N1/21 C07K14/195
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 30, 1992, pages 21873-21878, XP002031066 P.F. MARRERO ET AL.: "Effects of site-directed mutagenesis of the highly conserved aspartate residues in domain II of farnesyl diphosphate synthase activity" *see the whole article* -----	1-14	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C12N C07K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	16 May 1997	Marie, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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P : intermediate document

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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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